



**HAL**  
open science

## Covalently closed circular DNA: The ultimate therapeutic target for curing HBV infections

Maria Guadalupe Martinez, Anders Boyd, Emmanuel Combe, Barbara Testoni, Fabien Zoulim

### ► To cite this version:

Maria Guadalupe Martinez, Anders Boyd, Emmanuel Combe, Barbara Testoni, Fabien Zoulim. Covalently closed circular DNA: The ultimate therapeutic target for curing HBV infections. *Journal of Hepatology*, 2021, 75 (3), pp.706-717. 10.1016/j.jhep.2021.05.013 . hal-04012209

**HAL Id: hal-04012209**

**<https://hal.science/hal-04012209v1>**

Submitted on 22 Aug 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Covalently closed circular DNA: the ultimate therapeutic target for curing Hepatitis B virus**  
2 **infections**

3  
4 Maria Guadalupe Martinez<sup>1</sup>, Anders Boyd<sup>2</sup>, Emmanuel Combe<sup>1</sup>, Barbara Testoni<sup>1</sup> and Fabien  
5 Zoulim<sup>1,3,4</sup>

6  
7 <sup>1</sup>INSERM U1052, CNRS UMR-5286, Cancer Research Center of Lyon (CRCL), Lyon, 69008,  
8 France ; <sup>2</sup> Stichting HIV Monitoring, Amsterdam, the Netherlands, Department of Infectious  
9 Diseases, Research and Prevention, Public Health Service of Amsterdam, Amsterdam, the  
10 Netherlands ; <sup>3</sup>University of Lyon, Université Claude- Bernard (UCBL), 69008 Lyon, France ;  
11 <sup>4</sup>Hospices Civils de Lyon (HCL), 69002 Lyon, France

12  
13 **Corresponding Author**

14 Fabien Zoulim. 151 Cours Albert Thomas. 69424 Lyon. Tel : 33472681970. Fax :  
15 33472681971. fabien.zoulim@inserm.fr

16  
17 **Keywords**

18 cccDNA, HBV cure, chronic hepatitis B, direct-acting antivirals

19  
20 **Electronic word count:** 5050

21 **Number of figures and tables:** 3 Figures

22  
23 **Conflict of Interest**

24 BT and FZ declare a patent licensed by Hoffmann-La Roche. FZ received grants from Beam  
25 Therapeutics and Evotec. FZ received consulting fees from Aligos, Antios, Assembly  
26 Bioscience and Enochian. FZ received honoraria from Gilead and MYR Pharma as a  
27 consultant.

28  
29 **Financial Support**

30 This work was funded by the University of Lyon “Excellence laboratories” grant (Labex  
31 DevWeCan n° ANR-10-LABX-61) to FZ and Agence Nationale de Recherches sur le SIDA et les  
32 hépatites virales (ANRS) to FZ and BT. AB received grants from the ANRS and Sidaction, paid  
33 to his institution.

34  
35 **Author contribution**

36 MGM, AB, EC and BT wrote the manuscript. FZ defined the outline and extensively revised  
37 the manuscript.

38  
39 **Abstract**

40  
41 Current antiviral therapies, such as pegylated interferon alpha and nucleos(t)ide  
42 analogs effectively improve the patient’s quality of life. However, they are limited to control  
43 the infection but cannot cure infected hepatocytes. Complete HBV cure is hampered due to  
44 the lack of therapies that can directly affect the viral minichromosome, covalently closed  
45 circular DNA. Efforts currently in the pipeline for early clinical trials aim to achieve a  
46 functional cure, defined by the loss of Hepatitis B surface antigen and undetectable HBV  
47 DNA levels in serum. However, achieving a complete HBV cure requires therapies that can

48 directly affect the cccDNA pool, either for its degradation, lethal mutations or functional  
49 silencing. In this review we discuss cutting-edge technologies that could lead to non-  
50 cytolytic direct cccDNA targeting, curing infected hepatocytes.

51

52 **Abstract word count:** 126

53

54 **Key points**

55 • HBV infection leads to the establishment of a pool of viral cccDNA minichromosome  
56 in the nuclei of infected cells that is responsible for viral persistence.

57 • Current recommended therapies for HBV efficiently achieve viral suppression  
58 improving patient's quality of life; however, they are unable to eliminate the viral  
59 minichromosome and thus, to cure CHB.

60 • The next achievable goal of current therapeutic research is achieving a functional  
61 cure leading to suppression of circulating HBsAg, after a finite treatment.

62 • Therapies aiming at direct cccDNA targeting, either for its degradation, lethal  
63 mutation in main HBV proteins or transcriptional silencing are actively being  
64 explored.

65 • Full understanding of cccDNA biology remains essential to find potential druggable  
66 targets and achieve HBV targeted therapies.

67 • Promising direct cccDNA targeting approaches in preclinical studies aimed at  
68 reducing or silencing the viral minichromosome reservoir need to overcome delivery,  
69 safety and feasibility issues.

70

71

72

73

74

75

76

77

78

79

80

81

82

83

84

85

86

87

88

89

90

91

92

93

94

95  
96  
97  
98

## Introduction

99 Hepatitis B virus (HBV) can lead to acute and chronic infections accounting for more  
100 than 250 million people infected worldwide and over 780,000 deaths yearly. Approved  
101 therapies include pegylated interferon- $\alpha$  (PEG-IFN) and Nucleos(t)ide analogs (NAs). PEG-  
102 IFN leads to sustained HBV suppression, though this was observed only in a limited number  
103 of patients, its mechanism of action is still unclear and prolonged therapy is poorly  
104 tolerated[1]. NAs control HBV replication improving patient's clinical outcome and are  
105 better tolerated than PEG-IFN[2]. However, neither of these treatments achieve a complete  
106 HBV cure (undetectable serum HBsAg and serum and intracellular HBV DNA, including  
107 cccDNA clearance) or a functional cure (undetectable serum HBV DNA and serum HBsAg,  
108 with or without seroconversion, accompanied or not by cccDNA silencing)[3] and in most  
109 cases, life-long treatments are required. Achieving a functional HBV cure after a finite  
110 course of treatment, leading to low rates of HBV reactivation, thus eliminating life-long  
111 therapies, is the next goal for HBV therapies.

112 The major challenge to achieve an HBV cure is the persistence of covalently closed  
113 circular DNA (cccDNA) viral minichromosome, which is unaffected by current  
114 therapies[4][5][6][7]. cccDNA is HBV molecular reservoir and serves as the only  
115 transcriptional template for all viral RNAs, including pregenomic RNA (pgRNA), and contains  
116 four overlapping open reading frames (ORFs): S, C, P and X. These four overlapping ORFs  
117 encode 7 viral proteins: HBeAg (secreted protein), HBc (viral capsid protein), HBV POL/RT  
118 (polymerase reverse transcriptase), PreS1/PreS2/HBsAg (large, medium, and small surface  
119 envelope glycoproteins), and HBx (transcriptional activator). The pregenomic RNA (pgRNA)  
120 transcript serves as the substrate for reverse transcription into new viral genome, the  
121 relaxed-circular DNA (rcDNA)[8]. NAs block pgRNA reverse transcription, preventing  
122 formation of new rcDNA-containing virions. However, they do not prevent viral transcription  
123 from cccDNA and antigen production, which can be decreased to some extent with  
124 interferon- $\alpha$ . Only few copies of cccDNA are sufficient for infection rebound after treatment  
125 cessation; thus, life-long treatments are needed to maintain control of the infection[6].  
126 Although HBV DNA integration is not essential for the viral replication cycle, a byproduct  
127 generated during cccDNA formation, double stranded linear (dsl)DNA, can be integrated  
128 into the host genome[9]. S and part of HBx sequences can be found integrated into the host  
129 genome enabling the production of both HBsAg and a truncated, but potentially functional,  
130 HBx form[10][11].

131 Due to the fundamental role of cccDNA in HBV replication cycle, finding therapies  
132 that can directly target cccDNA is pivotal to develop a complete or functional cure for  
133 chronic hepatitis B (CHB), defined by cccDNA degradation, lethal mutations (e.g. early stop  
134 codons in main ORFs that lead to replication defective virus) or irreversible silencing. Based  
135 on current knowledge of cccDNA biology, strategies to directly target cccDNA will be  
136 discussed in this review.

137

### **1. HBV cccDNA**

139

#### **1.1 Biogenesis**

140  
141

142 After internalization via the cellular receptor sodium-taurocholate cotransporting  
143 polypeptide (NTCP)[12], HBV delivers its 3.2kb rcDNA genome into the nuclei of the host  
144 hepatocyte. Since none of the DNA strands in rcDNA is covalently closed, it has to be  
145 “repaired” into the fully double stranded (dsDNA), cccDNA.

146 The mechanism of rcDNA-to-cccDNA conversion is not fully understood, and its  
147 investigation has been hampered by the absence of robust methods to detect and quantify  
148 cccDNA in high-throughput approaches. Formation of cccDNA from rcDNA is a multistep  
149 process that begins with the removal of HBV POL leading to protein-free rcDNA (PF-rcDNA,  
150 aka deproteinated rcDNA (DP-rcDNA)) formation (Fig. 1). Initial studies suggested an  
151 essential role of tyrosyl-DNA phosphodiesterase 2 (TDP2) in this step[13]. However, other  
152 studies indicate that TDP2 may be dispensable thus, the role of TDP-2 in rcDNA-to-cccDNA  
153 formation remains controversial[14][15][16]. Subsequently, the DNA flap and RNA primer  
154 are removed leading to the final step of “repairing” the ssDNA gap in the plus strand,  
155 resulting in cccDNA. Several enzymes from the host repair system, including DNA pol  $\kappa$  and  
156  $\alpha$ [17][18], DNA ligases 1 and 3 (Lig 1 and 3)[19] and Flap endonuclease 1 (FEN-1)[20],  
157 provide all the activities required in this transformation. Noteworthy, five cellular factors  
158 involved in the DNA lagging strand synthesis are essential and sufficient for rcDNA-to-  
159 cccDNA conversion *in vitro*: proliferating cell nuclear antigen (PCNA), PCNA-loading  
160 replication factor (RFC) complex, Pol  $\delta$ , FEN-1 and Lig1[21]. Interestingly, recent evidence  
161 suggests differential repair of the minus and plus-strand of rcDNA requiring different sets of  
162 human repair factors[22]. Nonetheless, cccDNA formation *in vivo* may be more complex and  
163 require additional host factors and this remains to be studied.

164 NAs do not target the rcDNA-to-cccDNA conversion, thus reducing cccDNA levels using  
165 NAs treatment would only be achieved if the precursor of cccDNA, rcDNA, is completely  
166 deleted. However, since NAs treatment does not achieve complete suppression of reverse  
167 transcriptase activity, resulting in incomplete inhibition of rcDNA synthesis, the pool of  
168 cccDNA can still be replenished [23]. Therefore, elucidating the complete list of host factors  
169 required in the conversion of rcDNA-to-cccDNA and antivirals that inhibit more profoundly  
170 viral DNA synthesis are essential to find druggable viral targets that can effectively prevent  
171 cccDNA pool replenishment via nuclear import of rcDNA from the cytoplasm, e.g.  
172 intracellular recycling, or via *de novo* infection of new hepatocytes.

173

## 174 **1.2 cccDNA chromatinization and transcriptional regulation**

175

176 cccDNA is wrapped around nucleosomes containing core histones3 (H3), H4, H2A and  
177 H2B, and associated with viral core protein (HBc), HBx and host transcription factors in the  
178 nuclei of infected hepatocytes, forming a highly stable  
179 minichromosome[24][25][26][27][28]. The mechanism of chromatin compaction, histone  
180 deposition and epigenetic regulation of cccDNA are still ill-defined. Like cellular genes,  
181 cccDNA is subjected to the histone code undergoing epigenetic regulation and dynamic  
182 exchanges by histone modifiers, chromatin remodelers and transcription  
183 factors[29][30][31]. Epigenetic regulation of HBV gene expression involves H3/H4-  
184 acetylation or methylation (e.g. H3 lysine4 trimethylation) serving as activation marks,  
185 facilitating chromatin accessibility and allowing gene transcription. Conversely, histones  
186 hypoacetylation and/or methylation (e.g. H3 lysine27 trimethylation) leads to a more  
187 compact chromatin, silencing cccDNA transcription[30]. Accordingly, high viremia in CHB  
188 patients correlates with hyperacetylation of cccDNA-associated H3/H4, potentially opening

189 cccDNA chromatin allowing the access of transcription factors for efficient HBV  
190 transcription[31].

191 Viral proteins also have a role in cccDNA transcriptional activity. HBc is a structural  
192 component that increases cccDNA compaction[25], yet HBc *de novo* synthesis may not be  
193 required for cccDNA transcription and its role in cccDNA stability *in vitro* remains  
194 debated[32]. It is possible that HBc remains associated to rcDNA after nuclear uncoating,  
195 bypassing the need of *de novo* synthesis. cccDNA-associated HBx is essential for its  
196 transcriptional activity: mutants lacking HBx infect cells but its cccDNA is silenced[33]. This  
197 silencing correlates with cccDNA-associated H3-hypoacetylation and more compact/less  
198 accessible minichromosome. In the absence of HBx, the histone-lysine N-methyltransferase  
199 SET Domain bifurcated histone-lysine N-methyltransferase 1 (SETDB1) is involved in cccDNA  
200 silencing, and this can be overcome by reintroducing HBx[34]. Additionally, cccDNA can be  
201 recognized and silenced by the Structural Maintenance of Chromosomes complex 5/6  
202 (Smc5/6). HBx counteracts Smc5/6 action on cccDNA by promoting its degradation via its  
203 binding partner, DNA damage-binding protein 1 (DDB1): HBx acts as a scaffold allowing the  
204 assembly of HBx-DDB1-CRL4 complexes leading to ubiquitylation and degradation of the  
205 Smc5/6[35][36][37][38]. It is enigmatic how the first molecules of HBx are produced: it is  
206 possible that early in the infection, low cccDNA levels are not detected by Smc5/6, allowing  
207 a first transcriptional wave to produce HBx, efficiently downregulating Smc5/6.  
208 Alternatively, HBx mRNA molecules found in Dane particles[39] could be functional  
209 producing the first HBx molecules.

210 Besides being regulated by associated histones modifications cccDNA contains two or  
211 three CpG islands, depending on the HBV genotype, strategically placed in regulatory  
212 regions[40]. While rcDNA is mostly unmethylated in serum and cytosol, nuclear HBV DNA  
213 methylation varies and could be associated to transcriptional repression. CpG2, located in  
214 HBx promoter, is minimally methylated in active cccDNA transcription and methylation of  
215 CpG2 is associated with lower viremia and HBsAg production[41]. Thus, DNA methylation  
216 can potentially control cccDNA transcriptional activity.

217

### 218 **1.3 cccDNA intracellular maintenance**

219 Newly synthesized HBc forms nucleocapsids (NCs) that encapsidate pgRNA and allow  
220 RT to form rcDNA. Encapsidated rcDNA can be enveloped and released from the cells  
221 forming infectious Dane particles that can infect new cells, or they may recycle back to the  
222 nucleus where the rcDNA is transformed into cccDNA. Both phenomena lead to the  
223 replenishment of the nuclear cccDNA pool, either at the single cell level or by increasing the  
224 number of infected cells. It is accepted that nuclear levels of cccDNA are highly stable,  
225 however, single-molecule cccDNA maintenance remains controversial.

226 A static cccDNA maintenance model suggests that as rcDNA nuclear import and  
227 rcDNA-to-cccDNA conversion decline, cccDNA levels are maintained by repressing *de novo*  
228 cccDNA formation/degradation (Fig.2)[42][43][44]. Consistent with this hypothesis, NAs  
229 indirectly affecting cccDNA levels by inhibiting *de novo* production of rcDNA, lead to minor  
230 effects on cccDNA nuclear pool. Translational studies indicate that intrahepatic cccDNA  
231 levels vary across CHB phases, with 1-10 copies/cell in HBeAg-positive patients and 1-2 logs  
232 less in HBeAg-negative patients [45][46][47][48]. Observations from CHB patients under NAs  
233 treatment suggested that complete cccDNA clearance could take decades[5][23][49][47],  
234 with most of the patients under long-term therapy (>3 years), still harboring low levels of  
235 cccDNA in their liver (1-10 copies/10<sup>3</sup> cells) [23][5][4]. This suggests that nuclear import of

236 *de novo* synthesized rcDNA plays a minor role in the cccDNA pool maintenance and that  
237 nuclear cccDNA is highly stable[50].

238 Recent evidence defied the static cccDNA maintenance model and showed persistence  
239 of residual levels of HBV replication during NAs treatment implying maintenance of the  
240 cccDNA pool by renewal. Furthermore, CHB patient's serum with low viremia under NAs  
241 treatment, are still infectious in chimeric mice, suggesting that a dynamic turnover, i.e.  
242 constant cccDNA formation/degradation, could explain cccDNA levels maintenance  
243 (Fig.2)[43][51][52]. In this dynamic model, constant degradation and *de novo* synthesis of  
244 cccDNA maintain stable nuclear copy numbers[53]. Supporting this model, retrospective  
245 studies in patient liver biopsies treated with NAs estimated cccDNA turnover rate in the  
246 infected liver between 5-11 weeks[52]. The underlying mechanism of cccDNA clearance and  
247 maintenance remains unclear: cccDNA destruction could occur dependently[54][55][56] or  
248 independently of infected hepatocyte death[57][58][59].

249 Given the constraints preventing single cell cccDNA quantification *in vivo*, these  
250 studies presented data as an average of cccDNA molecules per cell in the infected liver,  
251 instead of the actual cccDNA copy number per infected hepatocyte or its temporal  
252 distribution, thus hampering the assessment of single nuclei cccDNA stability. Therefore, it is  
253 still currently unknown if recycling of rcDNA to form cccDNA is an infrequent event in  
254 response to cccDNA loss, or if it is a permanent event counteracting constant cccDNA  
255 degradation, *in vivo*.

256 cccDNA does not follow a semiconservative replication and is not tethered to  
257 chromosomes, thus its fate after cell division remains controversial. Studies in HBV-infected  
258 liver-humanized mice, in the context of increased hepatocyte proliferation and efficient  
259 inhibition of virus reinfection, showed a reduction of the cccDNA pool, suggesting cccDNA  
260 loss upon cell division[60]. cccDNA labeling by fluorescence imaging *in situ* hybridization in  
261 the presence of NAs suggested that cccDNA is asymmetrically distributed to daughter cells,  
262 instead of being lost or duplicated during cell division[61]. Thus, in the presence of NAs, loss  
263 of cccDNA relies on the rate of infected hepatocyte death and further dilution by cell  
264 division. Accordingly, recent *in vitro* data in HepG2-NTCP cells suggested that cccDNA  
265 turnover is linked to infected hepatocytes turnover[32].

266 Elimination of cccDNA by agents targeting its biogenesis may thus be possible if the  
267 reduction in cccDNA levels is faster than its generation and if new cccDNA formation is  
268 effectively blocked. Thus, understanding the turnover time and fate of cccDNA pool in  
269 infected hepatocytes is key in antiviral strategies design.

270

#### 271 **1.4 Modeling cccDNA kinetics in patients**

272

273 When a therapy intended to cure HBV is given to an individual, there is interest in  
274 determining the rate at which the cccDNA pool decreases, thereby providing some notion of  
275 when eradication can be achieved. This can be studied through modeling cccDNA levels as  
276 an exponential function of decay, from which we are able to estimate the half-life of cccDNA  
277 levels, i.e. the time taken to halve the cccDNA level at its initial value. Ideally, serial liver  
278 biopsies from individuals (or experimental animals) taken at several time points during  
279 treatment would be used to measure (1) cccDNA quantity through PCR-based assays and (2)  
280 the total number of cells per sample. cccDNA copies/cells would be modeled as a function  
281 over time, which has been done previously done in woodchucks[44] and ducks[62]. If serial  
282 samples are unavailable, the copies/cells could be measured cross-sectionally at different

283 time points and the same models applied. This was done in a previous study of HIV-HBV co-  
284 infected individuals to estimate the half-life of cccDNA during treatment with tenofovir  
285 disoproxil fumarate (TDF)[23].

286 However, these models assume a constant exponential cccDNA decline rate, which  
287 might not be the most appropriate function if levels stabilize at a certain point during  
288 treatment. These models are also unable to accommodate a number of other factors that  
289 might govern changes in cccDNA levels. For instance, impaired innate immunity[48],  
290 genome recycling during replication, secondary infections of neighboring hepatocytes[53]  
291 and clearance of infected hepatocytes[32] relate to the effectiveness of clearing cccDNA  
292 during therapy. There have been attempts to more thoroughly understand cccDNA half-life  
293 while simultaneously modeling the rates of these other “compartments”, namely cytotoxic  
294 T-lymphocytes (CTL) and non-CTL immune responses[63], integration of HBV DNA[64], and  
295 cell-to-cell transmission of viral particles[65]. These models usually involve sets of ordinary  
296 differential equations[66], but have rarely extended to modeling cccDNA levels during  
297 therapy. These models could be used to establish potential phases of cccDNA decline given  
298 the different compartments leading to cccDNA persistence, and the combinations of direct  
299 and indirect therapies that could lead to faster cccDNA declines. Still, they are limited by the  
300 large quantities of inputs, missing inputs and the large numbers of assumptions required.

301 Liver biopsy samples have become highly unpopular over the past decade, with non-  
302 invasive liver fibrosis becoming the norm, thus the ability to conduct a study with these  
303 ideal conditions is almost impossible. Alternative markers whose changes bear strong  
304 correlation with cccDNA changes could be used to shed light on cccDNA half-lives without  
305 the need for liver samples. Hepatitis B core-related antigen (HBcrAg) and circulating HBV  
306 RNAs are candidates to emulate these ideal conditions[67]. Interestingly, the proportion of  
307 LAM-resistant variants during LAM-treatment, according to HBV RNAs, was used as a close  
308 proxy for cccDNA pools turnover rate, and hence cccDNA half-life in HBeAg-positive  
309 patients. The observed half-lives in that study ranged between 6.9-21.7 weeks, which is  
310 much shorter than expected[52]. These estimations came from individuals who likely had  
311 high transaminase levels, which might bring way to more rapid changes in cccDNA levels.  
312 Whether such a proxy could be used to estimate cccDNA levels during HBeAg-negative  
313 infection, especially when HBV-DNA levels are undetectable and transaminase levels are  
314 low, is uncertain. Despite improvements in these proxies, their potential for measurement  
315 error remains problematic.

316

## 317 **2. cccDNA targeting**

318

### 319 **2.1 Targeting cccDNA formation**

320

321 cccDNA biosynthesis represents an attractive target to reduce the cccDNA pool in the  
322 nuclei of infected hepatocytes. However, understanding the complete list of host factors  
323 required in the rcDNA-to-cccDNA conversion is essential. One limitation for these studies is  
324 the functional redundancy among DNA repair components: residual levels of the targeted  
325 proteins, or their paralog(s), may be sufficient to execute their function. Notwithstanding,  
326 functional redundancy exists because these factors are essential in cell homeostasis, so their  
327 complete knock-down is unachievable and therefore they do not represent a potential  
328 therapeutic target *in vivo*. Hence, approaches that can specifically target the viral  
329 components or specific viral/host factors interaction, without affecting essential host



330 protein's function, are needed. Small molecules blocking rcDNA deproteination have been  
331 described, however their antiviral target and mechanism of action remains to be  
332 determined[68][69].

333

334

## 335 **2.2 Targeting established cccDNA**

336

### 337 **2.2.1 Direct cccDNA targeting.**

338

339 Achieving complete or functional HBV cure requires direct cccDNA targeting, thus  
340 strategies leading to its degradation, lethal mutations or irreversible silencing in a non-  
341 cytolytic manner are greatly needed. Recent efforts identified a novel small molecule that  
342 reduced cccDNA levels in HBV-infected primary human hepatocytes (PHHs) and in mouse  
343 liver after hydrodynamic injection with an HBV minicircle[70]. This data is encouraging,  
344 though its safety profile and mechanism of action still need to be elucidated to determine if  
345 it directly targets cccDNA, or if cccDNA decline is an indirect effect due to perturbation of  
346 cellular processes.

347 Gene-editing approaches can disrupt the viral genome in a permanent way (Fig.3):  
348 Zinc-Finger nucleases (ZFNs)[71] and transcriptional activator-like effector nucleases  
349 (TALENs)[72] target and cleave HBV DNA sequences. Target DNA analysis showed insertion-  
350 deletions (indels) in the target sequences, consistent with imprecise rejoining by the non-  
351 homologous end joining (NHEJ) repair system and a drop in the levels of viral parameters.  
352 Precision Bioscience's meganuclease-HBV program (HBV-ARCUS) targets the viral POL  
353 leading to durable antigen loss in Hep3B cells containing integrated copies of HBV genome  
354 and in HBV-infected PHHs (P-1057[73]). *In vivo* studies in an AAV-episomal mouse model  
355 showed robust editing in liver tissues. Being smaller than ZFNs and TALENs, meganucleases  
356 present an advantage towards their delivery. However, comprehensive screenings of  
357 efficient target sequences using meganucleases is difficult given their labor-intensive  
358 production, and thus only few selected target sequences were evaluated potentially missing  
359 more efficient target sites.

360 Since the discovery of CRISPR-Cas9 applications in the human genome, gene-editing  
361 programs have been advancing into clinics[74][75][76]. CRISPR-Cas9 can be redirected to  
362 DNA sequences by redesigning the guide RNAs (gRNAs) complementary to the desired  
363 target. Cas9 protein generates double stranded breaks (DSBs) that are often imperfectly  
364 repaired by the NHEJ, disrupting the target sequence. Several publications presented proof-  
365 of-concept that CRISPR-Cas9 can target HBV DNA[77]. However, different infection models  
366 lead to different outcomes, from HBV genome degradation to repair. Accessibility to cccDNA  
367 and outcomes can differ from plasmid transfection to integrated HBV genome or *de novo*  
368 synthesized cccDNA presenting differences in chromatin status[27]. CpG methylation can  
369 lead to recruitment of other factors, particularly in CpG islands, that could hinder Cas9  
370 binding[78]. Nucleosomes strongly impair Cas9 binding to target sequences in cell-free  
371 assays. However, *in vivo*, nucleosomes are dynamic and sequences can regain accessibility  
372 due to nucleosome remodeling and breathing, where nucleosomal DNA is in equilibrium  
373 between wrapped and unwrapped states, allowing protein access to binding sites hidden by  
374 nucleosomes[79].

375 Despite the interest in designer nuclease approaches, several considerations should  
376 be addressed before their clinical application in CHB. Our group recently showed that

377 CRISPR-Cas9 can target cccDNA in HBV-infected hepatocytes leading to cccDNA degradation  
378 and repair[80]. However, HBV DNA variants were generated after dual gRNA targeting  
379 highlighting the importance of understanding the fate of cccDNA after gene editing. CRISPR-  
380 Cas9 treatment targeting HBV in chronically infected liver-humanized mice undergoing NA  
381 treatment, showed HBV DNA editing in 5/8 treated mice: though there was a trend towards  
382 cccDNA levels reduction, there was no change in viremia after treatment, indicating that a  
383 large cccDNA fraction remained unaffected, leading to viral rebound and dampening any  
384 therapeutic effect[81]. Several factors will be instrumental for the clinical application of  
385 gene-editing approaches for HBV therapy. Maximizing gene-editing efficiency, which is  
386 connected to high Cas9 expression levels in target cells, is essential. However, pre-existing  
387 immunity to Cas9 could be a major impediment and understanding Cas proteins  
388 immunogenicity in humans is essential: data on this topic is mixed, showing that as low as  
389 10%-2.5%[82] up to 78%-58% [83] of tested individual have pre-existing anti-SaCas9  
390 antibodies (*S.aureus*) or anti-SpCas9 antibodies (*S.pyogenes*) respectively. Pre-existent  
391 immunity led to elimination of genome-edited cells in mice, however the outcome in  
392 humans is difficult to predict[84]. Off-targets are also a concern: the extent of these events  
393 is still unclear and better approaches to assess off-targets are needed. Another critical step  
394 is delivery: achieving long-lasting effects in the viral genome with low off-target rates will  
395 require efficient, and preferentially transient, delivery systems to target all infected  
396 hepatocytes[85]. The use of *in vitro* transcribed mRNAs to express the gene editors, as  
397 opposed to viral vectors, provides several advantages: viral vectors present a risk of  
398 recombination with host DNA and precise dose regulation from DNA expression cassettes  
399 poses additional concerns.

400 Nuclease approaches share a disadvantage when envisioning their use to target HBV  
401 genome: they all lead to DSBs. Given the high sequence similarity between cccDNA, rcDNA  
402 and integrated HBV DNA, using designer-nuclease approaches will generate DSBs in the host  
403 genome potentially risking genomic instability, chromosomal recombination and  
404 pathological consequences[86]. Conveniently, gene-editing tools are already showing a clear  
405 evolution as exemplified by base and prime editing. Both of these approaches lead to DNA  
406 rewriting without cleavage, allowing control of the sequence outcome and should reduce  
407 the risk of host genome rearrangements[87][88]. Yan *et al* presented first proof-of-concept  
408 that Cytosine Base Editors (CBEs) targeting S gene sequence lead to a reduction of HBsAg  
409 and extracellular HBV DNA in HepG2.2.15 and HBV-infected HepG2-NTCP-C4 cells[89]. These  
410 studies are encouraging as these approaches can potentially lead to safely target cccDNA  
411 and integrated HBV DNA (Fig3.B). However, further studies evaluating the direct effect of  
412 base editors on cccDNA and its fate after editing are essential[90].

413 Assuming the concerns regarding the use of gene/base editors to treat CHB will be  
414 resolved, and given the extensive number of people chronically infected with HBV, careful  
415 evaluation on the product development will be required to ensure unrestricted access to  
416 the treatment. In this regard, production of *in vitro* transcribed mRNA may provide an  
417 economical advantage, however development of mass-production technology at low cost  
418 will be fundamental.

419

## 420 **2.2.2 Targeting cellular factors that can directly affect cccDNA**

421

422 APOBEC3s are cellular defense mechanism that recognize and deaminate foreign DNA,  
423 leading to C-to-T/G-to-A hypermutations[91]. Human APOBEC3s are activated by cytokines

424 such as IFNs, produced upon detection of foreign DNA. APOBEC 3A and 3B (A3A and A3B)  
425 overexpression lead to deamination and destruction of HBV DNA (Fig3)[92], and were  
426 suggested to generate non-cytotoxic cccDNA deamination[93]. Accordingly, results in HBV-  
427 infected HepaRG cells and PHHs suggested that IFN and lymphotoxin beta receptor (LTβR)  
428 led to non-cytolytic cccDNA clearance due to APOBEC upregulation[57][93]. A3A/B  
429 upregulation by HBV-specific CTL-associated cytokines lead to cccDNA minus strand editing,  
430 resulting in mismatch mutations and cccDNA degradation[94]. CTLs can be engineered to  
431 target exclusively HBV-infected hepatocytes or hepatocytes producing antigens from HBV-  
432 DNA integrations[95]. Given that a substantial number of hepatocytes could be infected  
433 during CHB, CTLs could trigger uncontrolled liver damage[96]. Engineered HBV-specific CTLs  
434 lacking cytolytic activity suppressed HBV replication via activation of the intracellular  
435 LTBR/APOBEC3 pathway[97]. However, apoptosis and subsequent cell proliferation were  
436 observed upon treatment, enabling the possibility that the effect on cccDNA could be in part  
437 due to cell division leading to cccDNA dilution. Furthermore, APOBEC cytosine-deaminase  
438 activity preferentially targets single stranded (ssDNA), thus other replicative intermediates,  
439 rather than cccDNA, would be a more suitable substrate. The deamination pattern of A3A in  
440 HBV replicative intermediates suggested that rcDNA could be the preferential APOBEC  
441 substrate[94]. Supporting this hypothesis, genomic data from patients showed that  
442 deamination occurred preferentially in the single-stranded region of the HBV genome[98].  
443 Studies in *in vitro* infected hepatocytes showed that IFN-α treatment did not lead to cccDNA  
444 deamination by APOBECs, and instead suggested that G-A hypermutations in virions  
445 occurred independently of IFN-α[99]. Thus, while HBV suppression was clearly  
446 demonstrated, direct cccDNA targeting by APOBEC remains controversial.

447 Strategies to regulate gene expression, such as CRISPR activation (CRISPRa), lead to  
448 specific APOBEC overexpression providing a different tool to evaluate its effect in cccDNA  
449 (Fig3.B-C). Briefly, the promoter of the gene of interest is targeted by a gRNA, locally  
450 recruiting a dead Cas9 (dCas9) protein fused to an activation domain[100]. CRISPRa  
451 specifically activated A3A/3B expression, leading to deamination of foreign episomal DNA,  
452 dismissing foreign integrated DNA. However, evidence of A3A/B activation by CRISPRa direct  
453 effects on cccDNA is still lacking. A main drawback for CRISPRa is that overexpression of  
454 intracellular deaminases can lead to mutagenesis of the host genome and cancer  
455 development[101] and this should be carefully evaluated.

456

### 457 **2.3 cccDNA Epigenetic silencing**

458

459 cccDNA transcription is regulated by the host cell epigenetic machinery. IFN-α  
460 treatment leads to epigenetic changes in cccDNA-associated histones and recruitment of  
461 transcriptional repressors, which could at least partially explain the reduction in cccDNA  
462 transcriptional activity. Members of the tripartite motif (TRIM) family expressed upon IFN  
463 response, in particular TRIM22, inhibit HBV transcription and replication in a non-cytolytic  
464 manner[102]. Pro-inflammatory cytokines showed a direct effect on cccDNA transcriptional  
465 activity, without affecting cccDNA levels: IL-6 treatment reduced cccDNA-associated  
466 histones acetylation affecting cccDNA transcriptional activity [103], and both IL-1β and IL-6  
467 reduced HBV RNA levels more efficiently than IFN-α *in vitro*[104]. IL-4 and TGF-β1 affect  
468 HBV transcription and replication, however direct evidence on their effect on cccDNA is  
469 lacking[105][106]. Understanding the mechanisms and factors involved in cccDNA  
470 epigenetic regulation is essential for the development of therapeutic strategies: though

471 cccDNA levels would remain intact, complete transcription shutdown should stall HBV  
472 production leading to a functional cure[107].

473 cccDNA-associated histones can be directly targeted to silence cccDNA transcription.  
474 The HDAC SIRT2 inhibitor, AGK2, suppressed cccDNA transcription[108], however integrated  
475 HBV DNA transcription was enhanced under the same conditions suggesting different  
476 epigenetic regulation of integrated versus episomal HBV DNA. KDM5 histone demethylase  
477 inhibitor led to a global increase in chromatin histone methylation, inhibiting viral RNAs and  
478 antigen production in PHHs[109]. PRMT5 methyltransferase was suggested to preferentially  
479 regulate cccDNA versus host chromatin, potentially by interacting directly with HBc[110].  
480 Treatment with the histone acetyltransferase p300/CBP inhibitor, C646, reduced HBV  
481 transcription[27]. Though epigenetic modifiers led to an interesting decrease in viral  
482 parameters, they could also silence tumor suppressor genes or other essential genes,  
483 contributing to hepatocarcinogenesis[111], and thus the critical balance of epigenetic  
484 modifiers for cell homeostasis should be carefully evaluated.

485 HBx role in cccDNA transcriptional activity makes it an interesting therapeutic  
486 target[37]. siRNAs targeting HBx mRNA transiently reduced HBx levels, leading to Smc5/6  
487 complex reappearance[112]. Moreover, a recent study showed that silencing all viral  
488 transcripts using a combination of siRNAs and peg-IFN $\alpha$  substantially decreased HBx protein  
489 leading to Smc6 rebound *in vivo*[113]. Designer nucleases, introducing mutations in HBx  
490 protein, could provide a good strategy to achieve this goal. However, targeting integrated  
491 HBx DNA variants, which retain its ability to degrade Smc5/6, may be essential to achieve  
492 irreversible cccDNA silencing and thus non-DSBs leading approaches should be  
493 considered[112]. Epigenetic editors are also being explored to silence HBx expression:  
494 methylation of the HBx promoter using the catalytic domain of DNMT3a fused to ZF led to  
495 reduction of HBV transcription[114]. Small molecules targeting the HBx-DDB1 interaction  
496 have been tested as potential antivirals for HBV. Data from HBV-infected PHHs treated with  
497 the FDA-approved small molecule nitazoxanide (NTZ) suggested a modest effect on Smc5  
498 rebound and viral parameters and *in vitro* data suggests that NTZ targets the interphase of  
499 HBx-DDB1[115]. Pevonedistat (MLN4924) is a small-molecule inhibitor of NEDD8-activating  
500 enzyme E1 (NAE): NEDD8 is essential for cullin activation, blocking NEDD8 activation, which  
501 impairs the HBx-DDB1-ROC1 (CRL4) E3-ligase complex essential for HBx-dependent Smc5/6  
502 degradation, thus blocking cccDNA transcriptional activity[116]. However, MLN4924 could  
503 directly affect HBx, contributing to reduced HBV replication. Evidently, epigenetic regulation  
504 could be exploited to control HBV infection (Fig.3C) and drug repositioning of FDA-approved  
505 compounds is an interesting strategy to find novel antivirals. However, understanding their  
506 mechanism leading to reduction of viral parameters in the context of CHB remains essential.

507

### 508 **3. Perspectives**

509

510 Treatments that could non-cytopathically eradicate cccDNA lack specificity, adequate  
511 and effective delivery approaches and a complete overview of off-target effects, foreclosing  
512 their clinical development. Approaches aiming to achieve a functional cure, defined as  
513 serum viral DNA clearance and loss of Hepatitis B Surface antigen (HBsAg) with or without  
514 anti-HBs sero-conversion, are under development[117]. These therapies aim to evaluate  
515 therapeutic combinations to more effectively inhibit HBV replication and viral antigen  
516 production, and reinvigorate exhausted immune responses. These combinatorial strategies

517 should result in decreased rcDNA supply and elimination of infected cells, thereby tipping  
518 the balance toward cccDNA loss.

519 NAs and capsid assembly modulators (CAMs) combinations are being explored to  
520 achieve complete viral suppression: ongoing phase 2 results show continued decline in  
521 serum HBV DNA and pgRNA levels[118]. siRNAs and antisense oligonucleotides (ASOs) are  
522 also being explored in combination with NAs: siRNAs JNJ-3989[119], AB-729[120] or ASOs  
523 (GSK-836)[121] showed positive target engagement with decreasing HBsAg levels, which  
524 was sustained after treatment withdrawal in some patients. Results from longer term dual  
525 and triple therapy trials including a CAM[122] are eagerly awaited. Combination of the entry  
526 inhibitor bulevirtide with tenofovir and PEG-IFN $\alpha$  to treat chronic hepatitis B-Delta showed  
527 strong synergy and reduced HBsAg levels in a substantial number of patients[123],  
528 suggesting functional cure of HBV might be achievable. Similar results were obtained by  
529 triple combination of NAs, peg-IFN $\alpha$  and nucleic acid polymers[124], however studies in  
530 larger cohorts are essential to evaluate its safety and efficacy. Increasing evidence suggests  
531 that decreasing HBsAg levels is not enough to restore anti-HBV immune responses,  
532 therefore combinations of NAs and innate immunity boosters[125], NAs and therapeutic  
533 vaccines[126] and NAs in combination with checkpoint inhibitors and therapeutic  
534 vaccines[127] are currently under evaluation. In a scenario where persistence of  
535 transcriptionally active cccDNA remains the mainstay, sustained restoration of immune  
536 control would be needed. It may rely on complex combinations of viral replication and  
537 antigen expression inhibitors together with immune invigoration and stimulation by  
538 therapeutic vaccines[128].

539 Exciting approaches to directly target cccDNA include gene-editing[77][80], epigenetic  
540 suppression of cccDNA transcription[129] and stimulation of hepatocyte innate immunity  
541 signaling leading to lethal mutations in cccDNA via APOBEC nucleotide deamination[130].  
542 Envisioning the use of engineer nucleases as a therapy for HBV requires an in-depth study of  
543 their off-targets, effects of the DSBs when targeting integrated HBV DNA, efficient and  
544 preferentially specific delivery to infected hepatocytes and broad spectrum targeting of  
545 different viral strains. Editing and inactivation of the HBV genome through innate immune  
546 pathways stimulation, that could be accomplished through oral administration of small  
547 molecules, is promising but achieving a robust and safe anti-HBV immune activation  
548 requires further investigation. Epigenetic modulators could obviate the challenges of  
549 eliminating cccDNA, but lack of virus over host chromatin specificity still represents a major  
550 drawback for the use of these drugs. Furthermore, if cccDNA silencing is envisioned via HBx  
551 targeting, both cccDNA and integrated HBx should be disrupted to prevent HBx expression  
552 and lead to sustained Smc5/6 reappearance.

553 Basic studies of cccDNA biology to better understand its formation, maintenance and  
554 kinetics, its interplay with host chromatin, and careful experimental evaluation of novel  
555 targeted therapies are mandatory prior to proceeding to clinical evaluations. They will also  
556 have to show an optimal safety profile and efficiency to consider replacing or combining  
557 with current or novel approaches in clinical development. In particular, possible  
558 combination approaches with NAs, alleviating the load of replicative intermediates, could  
559 lead to more efficient cccDNA targeting. Thus, evidence on the synergistic effects and  
560 potential undesirable effects will have to be carefully evaluated.

## 561 REFERENCES

- 562 [1] Isorce N, Lucifora J, Zoulim F, Durantel D. Immune-modulators to combat hepatitis B  
563 virus infection: From IFN- $\alpha$  to novel investigational immunotherapeutic strategies.  
564 Antiviral Research 2015;122:69–81. <https://doi.org/10.1016/j.antiviral.2015.08.008>.
- 565 [2] Gish R, Jia J-D, Locarnini S, Zoulim F. Selection of chronic hepatitis B therapy with high  
566 barrier to resistance. The Lancet Infectious Diseases 2012;12:341–53.  
567 [https://doi.org/10.1016/S1473-3099\(11\)70314-0](https://doi.org/10.1016/S1473-3099(11)70314-0).
- 568 [3] Cornberg M, Lok AS-F, Terrault NA, Zoulim F, 2019 EASL-AASLD HBV Treatment  
569 Endpoints Conference Faculty. Guidance for design and endpoints of clinical trials in  
570 chronic hepatitis B - Report from the 2019 EASL-AASLD HBV Treatment Endpoints  
571 Conference. J Hepatol 2020;72:539–57. <https://doi.org/10.1016/j.jhep.2019.11.003>.
- 572 [4] Lebossé F, Inchauspé A, Locatelli M, Miaglia C, Diederichs A, Fresquet J, et al.  
573 Quantification and epigenetic evaluation of the residual pool of hepatitis B covalently  
574 closed circular DNA in long-term nucleoside analogue-treated patients. Sci Rep  
575 2020;10:21097. <https://doi.org/10.1038/s41598-020-78001-1>.
- 576 [5] Lai C-L, Wong D, Ip P, Kopaniszen M, Seto W-K, Fung J, et al. Reduction of covalently  
577 closed circular DNA with long-term nucleos(t)ide analogue treatment in chronic  
578 hepatitis B. J Hepatol 2017;66:275–81. <https://doi.org/10.1016/j.jhep.2016.08.022>.
- 579 [6] Lai C-L, Wong DK-H, Wong GT-Y, Seto W-K, Fung J, Yuen M-F. Rebound of HBV DNA  
580 after cessation of nucleos(t)ide analogues in chronic hepatitis B patients with  
581 undetectable covalently closed circular DNA. JHEP Reports 2020;2:100112.  
582 <https://doi.org/10.1016/j.jhepr.2020.100112>.
- 583 [7] Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of  
584 chronic hepatitis B. Gut 2015;64:1972–84. <https://doi.org/10.1136/gutjnl-2015-309809>.
- 585
- 586 [8] Hu J, Seeger C. Hepadnavirus Genome Replication and Persistence. Cold Spring Harb  
587 Perspect Med 2015;5:a021386. <https://doi.org/10.1101/cshperspect.a021386>.
- 588 [9] Tu T, Budzinska MA, Vondran FWR, Shackel NA, Urban S. Hepatitis B Virus DNA  
589 Integration Occurs Early in the Viral Life Cycle in an *In Vitro* Infection Model via  
590 Sodium Taurocholate Cotransporting Polypeptide-Dependent Uptake of Enveloped  
591 Virus Particles. Journal of Virology 2018;92:e02007-17.  
592 <https://doi.org/10.1128/JVI.02007-17>.
- 593 [10] Ali A. Hepatitis B virus, HBx mutants and their role in hepatocellular carcinoma. WJG  
594 2014;20:10238. <https://doi.org/10.3748/wjg.v20.i30.10238>.
- 595 [11] Wooddell CI, Yuen M-F, Chan HL-Y, Gish RG, Locarnini SA, Chavez D, et al. RNAi-based  
596 treatment of chronically infected patients and chimpanzees reveals that integrated  
597 hepatitis B virus DNA is a source of HBsAg. Sci Transl Med 2017;9.  
598 <https://doi.org/10.1126/scitranslmed.aan0241>.
- 599 [12] Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting  
600 polypeptide is a functional receptor for human hepatitis B and D virus. ELife 2012;1.  
601 <https://doi.org/10.7554/eLife.00049>.
- 602 [13] Königer C, Wingert I, Marsmann M, Rösler C, Beck J, Nassal M. Involvement of the  
603 host DNA-repair enzyme TDP2 in formation of the covalently closed circular DNA  
604 persistence reservoir of hepatitis B viruses. Proc Natl Acad Sci USA 2014;111:E4244–  
605 53. <https://doi.org/10.1073/pnas.1409986111>.

- 606 [14] Cui X, McAllister R, Boregowda R, Sohn JA, Ledesma FC, Caldecott KW, et al. Does  
607 Tyrosyl DNA Phosphodiesterase-2 Play a Role in Hepatitis B Virus Genome Repair?  
608 PLoS ONE 2015;10:e0128401. <https://doi.org/10.1371/journal.pone.0128401>.
- 609 [15] Winer BY, Huang TS, Pludwinski E, Heller B, Wojcik F, Lipkowitz GE, et al. Long-term  
610 hepatitis B infection in a scalable hepatic co-culture system. *Nat Commun* 2017;8:125.  
611 <https://doi.org/10.1038/s41467-017-00200-8>.
- 612 [16] Cai D, Yan R, Xu JZ, Zhang H, Shen S, Mitra B, et al. Characterization of the Termini of  
613 Cytoplasmic Hepatitis B Virus Deproteinized Relaxed Circular DNA. *J Virol*  
614 2020;95:e00922-20, /jvi/95/1/JVI.00922-20.atom. [https://doi.org/10.1128/JVI.00922-](https://doi.org/10.1128/JVI.00922-20)  
615 20.
- 616 [17] Qi Y, Gao Z, Xu G, Peng B, Liu C, Yan H, et al. DNA Polymerase  $\kappa$  Is a Key Cellular Factor  
617 for the Formation of Covalently Closed Circular DNA of Hepatitis B Virus. *PLoS Pathog*  
618 2016;12:e1005893. <https://doi.org/10.1371/journal.ppat.1005893>.
- 619 [18] Tang L, Sheraz M, McGrane M, Chang J, Guo J-T. DNA Polymerase alpha is essential  
620 for intracellular amplification of hepatitis B virus covalently closed circular DNA. *PLoS*  
621 *Pathog* 2019;15:e1007742. <https://doi.org/10.1371/journal.ppat.1007742>.
- 622 [19] Long Q, Yan R, Hu J, Cai D, Mitra B, Kim ES, et al. The role of host DNA ligases in  
623 hepadnavirus covalently closed circular DNA formation. *PLoS Pathog*  
624 2017;13:e1006784. <https://doi.org/10.1371/journal.ppat.1006784>.
- 625 [20] Kitamura K, Que L, Shimadu M, Koura M, Ishihara Y, Wakae K, et al. Flap  
626 endonuclease 1 is involved in cccDNA formation in the hepatitis B virus. *PLoS Pathog*  
627 2018;14:e1007124. <https://doi.org/10.1371/journal.ppat.1007124>.
- 628 [21] Wei L, Ploss A. Core components of DNA lagging strand synthesis machinery are  
629 essential for hepatitis B virus cccDNA formation. *Nat Microbiol* 2020;5:715–26.  
630 <https://doi.org/10.1038/s41564-020-0678-0>.
- 631 [22] Wei L, Ploss A. Hepatitis B virus cccDNA is formed through distinct repair processes of  
632 each strand. *Nat Commun* 2021;12:1591. [https://doi.org/10.1038/s41467-021-21850-](https://doi.org/10.1038/s41467-021-21850-9)  
633 9.
- 634 [23] Boyd A, Lacombe K, Lavocat F, Maylin S, Miallhes P, Lascoux-Combe C, et al. Decay of  
635 ccc-DNA marks persistence of intrahepatic viral DNA synthesis under tenofovir in HIV-  
636 HBV co-infected patients. *J Hepatol* 2016;65:683–91.  
637 <https://doi.org/10.1016/j.jhep.2016.05.014>.
- 638 [24] Bock CT, Schranz P, Schröder CH, Zentgraf H. Hepatitis B virus genome is organized  
639 into nucleosomes in the nucleus of the infected cell. *Virus Genes* 1994;8:215–29.
- 640 [25] Bock CT, Schwinn S, Locarnini S, Fyfe J, Manns MP, Trautwein C, et al. Structural  
641 organization of the hepatitis B virus minichromosome. *J Mol Biol* 2001;307:183–96.  
642 <https://doi.org/10.1006/jmbi.2000.4481>.
- 643 [26] Belloni L, Pollicino T, De Nicola F, Guerrieri F, Raffa G, Fanciulli M, et al. Nuclear HBx  
644 binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA  
645 function. *Proc Natl Acad Sci USA* 2009;106:19975–9.  
646 <https://doi.org/10.1073/pnas.0908365106>.
- 647 [27] Tropberger P, Mercier A, Robinson M, Zhong W, Ganem DE, Holdorf M. Mapping of  
648 histone modifications in episomal HBV cccDNA uncovers an unusual chromatin  
649 organization amenable to epigenetic manipulation. *Proc Natl Acad Sci USA*  
650 2015;112:E5715-5724. <https://doi.org/10.1073/pnas.1518090112>.
- 651 [28] Protzer U. Epigenetic control of HBV by HBx protein—releasing the break? *Nat Rev*  
652 *Gastroenterol Hepatol* 2015;12:558–9. <https://doi.org/10.1038/nrgastro.2015.152>.

- 653 [29] Bernstein BE, Meissner A, Lander ES. The Mammalian Epigenome. *Cell* 2007;128:669–  
654 81. <https://doi.org/10.1016/j.cell.2007.01.033>.
- 655 [30] Pollicino T, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G, et al. Hepatitis B  
656 virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-  
657 bound H3 and H4 histones. *Gastroenterology* 2006;130:823–37.  
658 <https://doi.org/10.1053/j.gastro.2006.01.001>.
- 659 [31] Hong X, Kim ES, Guo H. Epigenetic regulation of hepatitis B virus covalently closed  
660 circular DNA: Implications for epigenetic therapy against chronic hepatitis B: Hong,  
661 Kim, and Guo. *Hepatology* 2017;66:2066–77. <https://doi.org/10.1002/hep.29479>.
- 662 [32] Tu T, Zehnder B, Qu B, Urban S. De novo synthesis of Hepatitis B virus nucleocapsids is  
663 dispensable for the maintenance and transcriptional regulation of cccDNA. *JHEP*  
664 *Reports* 2020:100195. <https://doi.org/10.1016/j.jhepr.2020.100195>.
- 665 [33] Lucifora J, Arzberger S, Durantel D, Belloni L, Strubin M, Levrero M, et al. Hepatitis B  
666 virus X protein is essential to initiate and maintain virus replication after infection.  
667 *Journal of Hepatology* 2011;55:996–1003.  
668 <https://doi.org/10.1016/j.jhep.2011.02.015>.
- 669 [34] Rivière L, Gerossier L, Ducroux A, Dion S, Deng Q, Michel M-L, et al. HBx relieves  
670 chromatin-mediated transcriptional repression of hepatitis B viral cccDNA involving  
671 SETDB1 histone methyltransferase. *J Hepatol* 2015;63:1093–102.  
672 <https://doi.org/10.1016/j.jhep.2015.06.023>.
- 673 [35] Brown JS, Jackson SP. Ubiquitylation, neddylation and the DNA damage response.  
674 *Open Biol* 2015;5:150018. <https://doi.org/10.1098/rsob.150018>.
- 675 [36] Murphy CM, Xu Y, Li F, Nio K, Reszka-Blanco N, Li X, et al. Hepatitis B Virus X Protein  
676 Promotes Degradation of SMC5/6 to Enhance HBV Replication. *Cell Rep*  
677 2016;16:2846–54. <https://doi.org/10.1016/j.celrep.2016.08.026>.
- 678 [37] Decorsière A, Mueller H, van Breugel PC, Abdul F, Gerossier L, Beran RK, et al.  
679 Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor.  
680 *Nature* 2016;531:386–9. <https://doi.org/10.1038/nature17170>.
- 681 [38] Niu C, Livingston CM, Li L, Beran RK, Daffis S, Ramakrishnan D, et al. The Smc5/6  
682 Complex Restricts HBV when Localized to ND10 without Inducing an Innate Immune  
683 Response and Is Counteracted by the HBV X Protein Shortly after Infection. *PLoS ONE*  
684 2017;12:e0169648. <https://doi.org/10.1371/journal.pone.0169648>.
- 685 [39] Stadelmayer B, Diederichs A, Chapus F, Rivoire M, Neveu G, Alam A, et al. Full-length  
686 5'RACE identifies all major HBV transcripts in HBV-infected hepatocytes and patient  
687 serum. *Journal of Hepatology* 2020;73:40–51.  
688 <https://doi.org/10.1016/j.jhep.2020.01.028>.
- 689 [40] Kim J-W, Lee SH, Park YS, Hwang J-H, Jeong S-H, Kim N, et al. Replicative Activity of  
690 Hepatitis B Virus Is Negatively Associated with Methylation of Covalently Closed  
691 Circular DNA in Advanced Hepatitis B Virus Infection. *Intervirology* 2011;54:316–25.  
692 <https://doi.org/10.1159/000321450>.
- 693 [41] Zhang Y, Mao R, Yan R, Cai D, Zhang Y, Zhu H, et al. Transcription of Hepatitis B Virus  
694 Covalently Closed Circular DNA Is Regulated by CpG Methylation during Chronic  
695 Infection. *PLoS ONE* 2014;9:e110442. <https://doi.org/10.1371/journal.pone.0110442>.
- 696 [42] Lutgehetmann M, Volz T, Köpke A, Broja T, Tigges E, Lohse AW, et al. In vivo  
697 proliferation of hepadnavirus-infected hepatocytes induces loss of covalently closed  
698 circular DNA in mice. *Hepatology* 2010;52:16–24. <https://doi.org/10.1002/hep.23611>.



- 699 [43] Zhu Y, Yamamoto T, Cullen J, Saputelli J, Aldrich CE, Miller DS, et al. Kinetics of  
700 Hepadnavirus Loss from the Liver during Inhibition of Viral DNA Synthesis. *J Virol*  
701 2001;75:311–22. <https://doi.org/10.1128/JVI.75.1.311-322.2001>.
- 702 [44] Dandri M, Burda MR, Will H, Petersen J. Increased hepatocyte turnover and inhibition  
703 of woodchuck hepatitis B virus replication by adefovir in vitro do not lead to reduction  
704 of the closed circular DNA. *Hepatology* 2000;32:139–46.  
705 <https://doi.org/10.1053/jhep.2000.8701>.
- 706 [45] Laras A, Koskinas J, Dimou E, Kostamena A, Hadziyannis SJ. Intrahepatic levels and  
707 replicative activity of covalently closed circular hepatitis B virus DNA in chronically  
708 infected patients. *Hepatology* 2006;44:694–702. <https://doi.org/10.1002/hep.21299>.
- 709 [46] Volz T, Lutgehetmann M, Wachtler P, Jacob A, Quaas A, Murray JM, et al. Impaired  
710 Intrahepatic Hepatitis B Virus Productivity Contributes to Low Viremia in Most HBeAg-  
711 Negative Patients. *Gastroenterology* 2007;133:843–52.  
712 <https://doi.org/10.1053/j.gastro.2007.06.057>.
- 713 [47] Werle-Lapostolle B, Bowden S, Locarnini S, Wursthorn K, Petersen J, Lau G, et al.  
714 Persistence of cccDNA during the natural history of chronic hepatitis B and decline  
715 during adefovir dipivoxil therapy. *Gastroenterology* 2004;126:1750–8.
- 716 [48] Lebossé F, Testoni B, Fresquet J, Facchetti F, Galmozzi E, Fournier M, et al.  
717 Intrahepatic innate immune response pathways are downregulated in untreated  
718 chronic hepatitis B. *Journal of Hepatology* 2017;66:897–909.  
719 <https://doi.org/10.1016/j.jhep.2016.12.024>.
- 720 [49] Gordon SC, Krastev Z, Horban A, Petersen J, Sperl J, Dinh P, et al. Efficacy of tenofovir  
721 disoproxil fumarate at 240 weeks in patients with chronic hepatitis B with high  
722 baseline viral load: *Hepatology*. *Hepatology* 2013;58:505–13.  
723 <https://doi.org/10.1002/hep.26277>.
- 724 [50] Moraleda G, Saputelli J, Aldrich CE, Averett D, Condreay L, Mason WS. Lack of effect  
725 of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of  
726 woodchuck hepatitis virus. *Journal of Virology* 1997;71:9392–9.  
727 <https://doi.org/10.1128/JVI.71.12.9392-9399.1997>.
- 728 [51] Burdette D, Cathcart A, Shauf A, Win R, Zaboli S, Hedskog C, et al. PS-150-Evidence for  
729 the presence of infectious virus in the serum from chronic hepatitis B patients  
730 suppressed on nucleos (t)ide therapy with detectable but not quantifiable HBV DNA.  
731 *Journal of Hepatology* 2019;70:e95. [https://doi.org/10.1016/S0618-8278\(19\)30168-9](https://doi.org/10.1016/S0618-8278(19)30168-9).
- 732 [52] Huang Q, Zhou B, Cai D, Zong Y, Wu Y, Liu S, et al. Rapid Turnover of Hepatitis B Virus  
733 Covalently Closed Circular DNA Indicated by Monitoring Emergence and Reversion of  
734 Signature-Mutation in Treated Chronic Hepatitis B Patients. *Hepatology* 2021;73:41–  
735 52. <https://doi.org/10.1002/hep.31240>.
- 736 [53] Ko C, Chakraborty A, Chou W-M, Hasreiter J, Wettengel JM, Stadler D, et al. Hepatitis  
737 B virus genome recycling and de novo secondary infection events maintain stable  
738 cccDNA levels. *Journal of Hepatology* 2018;69:1231–41.  
739 <https://doi.org/10.1016/j.jhep.2018.08.012>.
- 740 [54] Guo J-T, Zhou H, Liu C, Aldrich C, Saputelli J, Whitaker T, et al. Apoptosis and  
741 Regeneration of Hepatocytes during Recovery from Transient Hepadnavirus  
742 Infections. *J Virol* 2000;74:1495–505. <https://doi.org/10.1128/JVI.74.3.1495-1505.2000>.
- 744 [55] Summers J, Jilbert AR, Yang W, Aldrich CE, Saputelli J, Litwin S, et al. Hepatocyte  
745 turnover during resolution of a transient hepadnaviral infection. *Proceedings of the*

- 746 National Academy of Sciences 2003;100:11652–9.  
747 <https://doi.org/10.1073/pnas.1635109100>.
- 748 [56] Mason WS, Jilbert AR, Summers J. Clonal expansion of hepatocytes during chronic  
749 woodchuck hepatitis virus infection. *Proceedings of the National Academy of Sciences*  
750 2005;102:1139–44. <https://doi.org/10.1073/pnas.0409332102>.
- 751 [57] Guidotti LG. Viral Clearance Without Destruction of Infected Cells During Acute HBV  
752 Infection. *Science* 1999;284:825–9. <https://doi.org/10.1126/science.284.5415.825>.
- 753 [58] Wieland SF, Spangenberg HC, Thimme R, Purcell RH, Chisari FV. Expansion and  
754 contraction of the hepatitis B virus transcriptional template in infected chimpanzees.  
755 *Proc Natl Acad Sci USA* 2004;101:2129–34.  
756 <https://doi.org/10.1073/pnas.0308478100>.
- 757 [59] Murray JM, Wieland SF, Purcell RH, Chisari FV. Dynamics of hepatitis B virus clearance  
758 in chimpanzees. *Proceedings of the National Academy of Sciences* 2005;102:17780–5.  
759 <https://doi.org/10.1073/pnas.0508913102>.
- 760 [60] Allweiss L, Volz T, Giersch K, Kah J, Raffa G, Petersen J, et al. Proliferation of primary  
761 human hepatocytes and prevention of hepatitis B virus reinfection efficiently deplete  
762 nuclear cccDNA in vivo. *Gut* 2018;67:542–52. <https://doi.org/10.1136/gutjnl-2016-312162>.
- 764 [61] Li M, Sohn JA, Seeger C. Distribution of Hepatitis B Virus Nuclear DNA. *J Virol*  
765 2017;92:e01391-17. <https://doi.org/10.1128/JVI.01391-17>.
- 766 [62] Addison WR, Walters K-A, Wong WWS, Wilson JS, Madej D, Jewell LD, et al. Half-life of  
767 the duck hepatitis B virus covalently closed circular DNA pool in vivo following  
768 inhibition of viral replication. *J Virol* 2002;76:6356–63.  
769 <https://doi.org/10.1128/jvi.76.12.6356-6363.2002>.
- 770 [63] Murray JM, Goyal A. In silico single cell dynamics of hepatitis B virus infection and  
771 clearance. *Journal of Theoretical Biology* 2015;366:91–102.  
772 <https://doi.org/10.1016/j.jtbi.2014.11.020>.
- 773 [64] Goyal A, Chauhan R. The dynamics of integration, viral suppression and cell-cell  
774 transmission in the development of occult Hepatitis B virus infection. *Journal of*  
775 *Theoretical Biology* 2018;455:269–80. <https://doi.org/10.1016/j.jtbi.2018.06.020>.
- 776 [65] Goyal A, Murray JM. Modelling the Impact of Cell-To-Cell Transmission in Hepatitis B  
777 Virus. *PLoS ONE* 2016;11:e0161978. <https://doi.org/10.1371/journal.pone.0161978>.
- 778 [66] Goyal A, Liao LE, Perelson AS. Within-host mathematical models of hepatitis B virus  
779 infection: Past, present, and future. *Current Opinion in Systems Biology* 2019;18:27–  
780 35. <https://doi.org/10.1016/j.coisb.2019.10.003>.
- 781 [67] Charre C, Levrero M, Zoulim F, Scholtès C. Non-invasive biomarkers for chronic  
782 hepatitis B virus infection management. *Antiviral Res* 2019;169:104553.  
783 <https://doi.org/10.1016/j.antiviral.2019.104553>.
- 784 [68] Cai D, Mills C, Yu W, Yan R, Aldrich CE, Saputelli JR, et al. Identification of  
785 disubstituted sulfonamide compounds as specific inhibitors of hepatitis B virus  
786 covalently closed circular DNA formation. *Antimicrob Agents Chemother*  
787 2012;56:4277–88. <https://doi.org/10.1128/AAC.00473-12>.
- 788 [69] Liu C, Cai D, Zhang L, Tang W, Yan R, Guo H, et al. Identification of hydrolyzable  
789 tannins (punicalagin, punicalin and geraniin) as novel inhibitors of hepatitis B virus  
790 covalently closed circular DNA. *Antiviral Research* 2016;134:97–107.  
791 <https://doi.org/10.1016/j.antiviral.2016.08.026>.

- 792 [70] Wang L, Zhu Q, Zeng J, Yan Z, Feng A, Young J, et al. PS-074-A first-in-class orally  
793 available HBV cccDNA destabilizer ccc\_R08 achieved sustainable HBsAg and HBV DNA  
794 suppression in the HBV circle mouse model through elimination of cccDNA-like  
795 molecules in the mouse liver. *Journal of Hepatology* 2019;70:e48.  
796 [https://doi.org/10.1016/S0618-8278\(19\)30086-6](https://doi.org/10.1016/S0618-8278(19)30086-6).
- 797 [71] Cradick TJ, Keck K, Bradshaw S, Jamieson AC, McCaffrey AP. Zinc-finger Nucleases as a  
798 Novel Therapeutic Strategy for Targeting Hepatitis B Virus DNAs. *Molecular Therapy*  
799 2010;18:947–54. <https://doi.org/10.1038/mt.2010.20>.
- 800 [72] Bloom K, Ely A, Mussolino C, Cathomen T, Arbuthnot P. Inactivation of Hepatitis B  
801 Virus Replication in Cultured Cells and In Vivo with Engineered Transcription  
802 Activator-Like Effector Nucleases. *Molecular Therapy* 2013;21:1889–97.  
803 <https://doi.org/10.1038/mt.2013.170>.
- 804 [73] 2020 ASGCT Annual Meeting Abstracts. *Molecular Therapy* 2020;28:1–592.  
805 <https://doi.org/10.1016/j.ymthe.2020.04.019>.
- 806 [74] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A Programmable  
807 Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science*  
808 2012;337:816–21. <https://doi.org/10.1126/science.1225829>.
- 809 [75] Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using  
810 the CRISPR-Cas9 system. *Nat Protoc* 2013;8:2281–308.  
811 <https://doi.org/10.1038/nprot.2013.143>.
- 812 [76] Ledford H, Callaway E. Pioneers of revolutionary CRISPR gene editing win chemistry  
813 Nobel. *Nature* 2020;586:346–7. <https://doi.org/10.1038/d41586-020-02765-9>.
- 814 [77] Bloom K, Maepa M, Ely A, Arbuthnot P. Gene Therapy for Chronic HBV—Can We  
815 Eliminate cccDNA? *Genes* 2018;9:207. <https://doi.org/10.3390/genes9040207>.
- 816 [78] Verkuijl SA, Rots MG. The influence of eukaryotic chromatin state on CRISPR–Cas9  
817 editing efficiencies. *Current Opinion in Biotechnology* 2019;55:68–73.  
818 <https://doi.org/10.1016/j.copbio.2018.07.005>.
- 819 [79] Isaac RS, Jiang F, Doudna JA, Lim WA, Narlikar GJ, Almeida R. Nucleosome breathing  
820 and remodeling constrain CRISPR-Cas9 function. *ELife* 2016;5:e13450.  
821 <https://doi.org/10.7554/eLife.13450>.
- 822 [80] Martinez MG, Inchauspe A, Delberghe E, Chapus F, Neveu G, Alam A, et al. SAT376 -  
823 Targeting hepatitis B virus with CRISPR/Cas9 approach. *Journal of Hepatology*  
824 2020;73:S841–2. [https://doi.org/10.1016/S0168-8278\(20\)32126-7](https://doi.org/10.1016/S0168-8278(20)32126-7).
- 825 [81] Stone D, Long KR, Loprieno MA, De Silva Felixge HS, Kenkel EJ, Liley RM, et al.  
826 CRISPR-Cas9 gene editing of hepatitis B virus in chronically infected humanized mice.  
827 *Molecular Therapy - Methods & Clinical Development* 2021;20:258–75.  
828 <https://doi.org/10.1016/j.omtm.2020.11.014>.
- 829 [82] Simhadri VL, McGill J, McMahon S, Wang J, Jiang H, Sauna ZE. Prevalence of Pre-  
830 existing Antibodies to CRISPR-Associated Nuclease Cas9 in the USA Population.  
831 *Molecular Therapy - Methods & Clinical Development* 2018;10:105–12.  
832 <https://doi.org/10.1016/j.omtm.2018.06.006>.
- 833 [83] Charlesworth CT, Deshpande PS, Dever DP, Camarena J, Lemgart VT, Cromer MK, et  
834 al. Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nat*  
835 *Med* 2019;25:249–54. <https://doi.org/10.1038/s41591-018-0326-x>.
- 836 [84] Li A, Tanner MR, Lee CM, Hurley AE, De Giorgi M, Jarrett KE, et al. AAV-CRISPR Gene  
837 Editing Is Negated by Pre-existing Immunity to Cas9. *Molecular Therapy*  
838 2020;28:1432–41. <https://doi.org/10.1016/j.ymthe.2020.04.017>.

- 839 [85] Rouet R, de Oñate L, Li J, Murthy N, Wilson RC. Engineering CRISPR-Cas9 RNA-Protein  
840 Complexes for Improved Function and Delivery. *The CRISPR Journal* 2018;1:367–78.  
841 <https://doi.org/10.1089/crispr.2018.0037>.
- 842 [86] Kosicki M, Tomberg K, Bradley A. Repair of double-strand breaks induced by CRISPR-  
843 Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol*  
844 2018;36:765–71. <https://doi.org/10.1038/nbt.4192>.
- 845 [87] Rees HA, Liu DR. Base editing: precision chemistry on the genome and transcriptome  
846 of living cells. *Nat Rev Genet* 2018;19:770–88. [https://doi.org/10.1038/s41576-018-](https://doi.org/10.1038/s41576-018-0059-1)  
847 [0059-1](https://doi.org/10.1038/s41576-018-0059-1).
- 848 [88] Anzalone AV, Koblan LW, Liu DR. Genome editing with CRISPR-Cas nucleases, base  
849 editors, transposases and prime editors. *Nat Biotechnol* 2020;38:824–44.  
850 <https://doi.org/10.1038/s41587-020-0561-9>.
- 851 [89] Yang Y-C, Chen Y-H, Kao J-H, Ching C, Liu I-J, Wang C-C, et al. Permanent Inactivation  
852 of HBV Genomes by CRISPR/Cas9-Mediated Non-cleavage Base Editing. *Molecular*  
853 *Therapy - Nucleic Acids* 2020;20:480–90.  
854 <https://doi.org/10.1016/j.omtn.2020.03.005>.
- 855 [90] Yu Y, Leete TC, Born DA, Young L, Barrera LA, Lee S-J, et al. Cytosine base editors with  
856 minimized unguided DNA and RNA off-target events and high on-target activity. *Nat*  
857 *Commun* 2020;11:2052. <https://doi.org/10.1038/s41467-020-15887-5>.
- 858 [91] Love RP, Xu H, Chelico L. Biochemical Analysis of Hypermutation by the Deoxycytidine  
859 Deaminase APOBEC3A. *J Biol Chem* 2012;287:30812–22.  
860 <https://doi.org/10.1074/jbc.M112.393181>.
- 861 [92] Stenglein MD, Burns MB, Li M, Lengyel J, Harris RS. APOBEC3 proteins mediate the  
862 clearance of foreign DNA from human cells. *Nat Struct Mol Biol* 2010;17:222–9.  
863 <https://doi.org/10.1038/nsmb.1744>.
- 864 [93] Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and  
865 nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science*  
866 2014;343:1221–8. <https://doi.org/10.1126/science.1243462>.
- 867 [94] Nair S, Zlotnick A. Asymmetric Modification of Hepatitis B Virus (HBV) Genomes by an  
868 Endogenous Cytidine Deaminase inside HBV Cores Informs a Model of Reverse  
869 Transcription. *J Virol* 2018;92:e02190-17, /jvi/92/10/e02190-17.atom.  
870 <https://doi.org/10.1128/JVI.02190-17>.
- 871 [95] Gehring AJ, Xue S-A, Ho ZZ, Teoh D, Ruedl C, Chia A, et al. Engineering virus-specific T  
872 cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. *J*  
873 *Hepatol* 2011;55:103–10. <https://doi.org/10.1016/j.jhep.2010.10.025>.
- 874 [96] Bertoletti A, Brunetto M, Maini MK, Bonino F, Qasim W, Stauss H. T cell receptor-  
875 therapy in HBV-related hepatocellular carcinoma. *Oncol Immunology* 2015;4:e1008354.  
876 <https://doi.org/10.1080/2162402X.2015.1008354>.
- 877 [97] Koh S, Kah J, Tham CYL, Yang N, Ceccarello E, Chia A, et al. Nonlytic Lymphocytes  
878 Engineered to Express Virus-Specific T-Cell Receptors Limit HBV Infection by  
879 Activating APOBEC3. *Gastroenterology* 2018;155:180-193.e6.  
880 <https://doi.org/10.1053/j.gastro.2018.03.027>.
- 881 [98] Suspene R, Guetard D, Henry M, Sommer P, Wain-Hobson S, Vartanian J-P. Extensive  
882 editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro  
883 and in vivo. *Proceedings of the National Academy of Sciences* 2005;102:8321–6.  
884 <https://doi.org/10.1073/pnas.0408223102>.

- 885 [99] Seeger C, Sohn JA. Complete Spectrum of CRISPR/Cas9-induced Mutations on HBV  
886 cccDNA. *Mol Ther* 2016;24:1258–66. <https://doi.org/10.1038/mt.2016.94>.
- 887 [100] Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, et al.  
888 Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from  
889 promoters and enhancers. *Nat Biotechnol* 2015;33:510–7.  
890 <https://doi.org/10.1038/nbt.3199>.
- 891 [101] Roberts SA, Lawrence MS, Klimczak LJ, Grimm SA, Fargo D, Stojanov P, et al. An  
892 APOBEC cytidine deaminase mutagenesis pattern is widespread in human cancers.  
893 *Nat Genet* 2013;45:970–6. <https://doi.org/10.1038/ng.2702>.
- 894 [102] Gao B, Duan Z, Xu W, Xiong S. Tripartite motif-containing 22 inhibits the activity of  
895 hepatitis B virus core promoter, which is dependent on nuclear-located RING domain.  
896 *Hepatology* 2009;50:424–33. <https://doi.org/10.1002/hep.23011>.
- 897 [103] Palumbo GA, Scisciani C, Pediconi N, Lupacchini L, Alfalate D, Guerrieri F, et al. IL6  
898 Inhibits HBV Transcription by Targeting the Epigenetic Control of the Nuclear cccDNA  
899 Minichromosome. *PLoS ONE* 2015;10:e0142599.  
900 <https://doi.org/10.1371/journal.pone.0142599>.
- 901 [104] Isorce N, Testoni B, Locatelli M, Fresquet J, Rivoire M, Luangsay S, et al. Antiviral  
902 activity of various interferons and pro-inflammatory cytokines in non-transformed  
903 cultured hepatocytes infected with hepatitis B virus. *Antiviral Research* 2016;130:36–  
904 45. <https://doi.org/10.1016/j.antiviral.2016.03.008>.
- 905 [105] Lin S-J, Shu P-Y, Chang C, Ng A-K, Hu C. IL-4 Suppresses the Expression and the  
906 Replication of Hepatitis B Virus in the Hepatocellular Carcinoma Cell Line Hep3B. *J*  
907 *Immunol* 2003;171:4708–16. <https://doi.org/10.4049/jimmunol.171.9.4708>.
- 908 [106] Hong M-H, Chou Y-C, Wu Y-C, Tsai K-N, Hu C, Jeng K-S, et al. Transforming Growth  
909 Factor- $\beta$ 1 Suppresses Hepatitis B Virus Replication by the Reduction of Hepatocyte  
910 Nuclear Factor-4 $\alpha$  Expression. *PLoS ONE* 2012;7:e30360.  
911 <https://doi.org/10.1371/journal.pone.0030360>.
- 912 [107] Mitra B, Thapa RJ, Guo H, Block TM. Host functions used by hepatitis B virus to  
913 complete its life cycle: Implications for developing host-targeting agents to treat  
914 chronic hepatitis B. *Antiviral Research* 2018;158:185–98.  
915 <https://doi.org/10.1016/j.antiviral.2018.08.014>.
- 916 [108] Yu H-B, Jiang H, Cheng S-T, Hu Z-W, Ren J-H, Chen J. AGK2, A SIRT2 Inhibitor, Inhibits  
917 Hepatitis B Virus Replication *In Vitro* And *In Vivo*. *International Journal of Medical*  
918 *Sciences* 2018;15:1356–64. <https://doi.org/10.7150/ijms.26125>.
- 919 [109] Gilmore S, Tam D, Dick R, Appleby T, Birkus G, Willkom M, et al. Antiviral activity of  
920 GS-5801, a liver-targeted prodrug of a lysine demethylase 5 inhibitor, in a hepatitis B  
921 virus primary human hepatocyte infection model. *Journal of Hepatology*  
922 2017;66:S690–1. [https://doi.org/10.1016/S0168-8278\(17\)31855-X](https://doi.org/10.1016/S0168-8278(17)31855-X).
- 923 [110] Zhang W, Chen J, Wu M, Zhang X, Zhang M, Yue L, et al. PRMT5 restricts hepatitis B  
924 virus replication through epigenetic repression of covalently closed circular DNA  
925 transcription and interference with pregenomic RNA encapsidation. *Hepatology*  
926 2017;66:398–415. <https://doi.org/10.1002/hep.29133>.
- 927 [111] Fernández-Barrena MG, Arechederra M, Colyn L, Berasain C, Avila MA. Epigenetics in  
928 hepatocellular carcinoma development and therapy: The tip of the iceberg. *JHEP*  
929 *Reports* 2020;2:100167. <https://doi.org/10.1016/j.jhepr.2020.100167>.
- 930 [112] Korniyev D, Ramakrishnan D, Voitenleitner C, Livingston CM, Xing W, Hung M, et al.  
931 Spatiotemporal Analysis of Hepatitis B Virus X Protein in Primary Human Hepatocytes.

- 932 J Virol 2019;93:e00248-19, /jvi/93/16/JVI.00248-19.atom.  
933 <https://doi.org/10.1128/JVI.00248-19>.
- 934 [113] Allweiss L, Giersch K, Piroso A, Volz T, Muench RC, Beran RK, et al. Therapeutic  
935 shutdown of HBV transcripts promotes reappearance of the SMC5/6 complex and  
936 silencing of the viral genome in vivo. *Gut* 2021;gutjnl-2020-322571.  
937 <https://doi.org/10.1136/gutjnl-2020-322571>.
- 938 [114] Xirong L, Rui L, Xiaoli Y, Qiuyan H, Bikui T, Sibó Z, et al. Hepatitis B virus can be  
939 inhibited by DNA methyltransferase 3a via specific zinc-finger-induced methylation of  
940 the X promoter. *Biochemistry Mosc* 2014;79:111–23.  
941 <https://doi.org/10.1134/S0006297914020047>.
- 942 [115] Sekiba K, Otsuka M, Ohno M, Yamagami M, Kishikawa T, Suzuki T, et al. Inhibition of  
943 HBV Transcription From cccDNA With Nitazoxanide by Targeting the HBx–DDB1  
944 Interaction. *Cellular and Molecular Gastroenterology and Hepatology* 2019;7:297–  
945 312. <https://doi.org/10.1016/j.jcmgh.2018.10.010>.
- 946 [116] Sekiba K, Otsuka M, Ohno M, Yamagami M, Kishikawa T, Seimiya T, et al.  
947 Pevonedistat, a Neuronal Precursor Cell-Expressed Developmentally Down-Regulated  
948 Protein 8–Activating Enzyme Inhibitor, Is a Potent Inhibitor of Hepatitis B Virus.  
949 *Hepatology* 2019;69:1903–15. <https://doi.org/10.1002/hep.30491>.
- 950 [117] Fanning GC, Zoulim F, Hou J, Bertolotti A. Therapeutic strategies for hepatitis B virus  
951 infection: towards a cure. *Nat Rev Drug Discov* 2019;18:827–44.  
952 <https://doi.org/10.1038/s41573-019-0037-0>.
- 953 [118] Yuen M-F, Agarwal K, Ma X, Nguyen T, Schiff ER, Hann H-W, et al. Antiviral activity and  
954 safety of the hepatitis B core inhibitor ABI-H0731 administered with a nucleos(t)ide  
955 reverse transcriptase inhibitor in patients with HBeAg-positive chronic hepatitis B  
956 infection in a long-term extension study. *Journal of Hepatology* 2020;73:S140.  
957 [https://doi.org/10.1016/S0168-8278\(20\)30790-X](https://doi.org/10.1016/S0168-8278(20)30790-X).
- 958 [119] Gane E, Locarnini S, Lim TH, Strasser S, Sievert W, Cheng W, et al. Short-term  
959 treatment with RNA interference therapy, JNJ-3989, results in sustained hepatitis B  
960 surface antigen suppression in patients with chronic hepatitis B receiving nucleos(t)ide  
961 analogue treatment. *Journal of Hepatology* 2020;73:S20.  
962 [https://doi.org/10.1016/S0168-8278\(20\)30597-3](https://doi.org/10.1016/S0168-8278(20)30597-3).
- 963 [120] Oral Abstracts. *Hepatology* 2020;72:1A-130A. <https://doi.org/10.1002/hep.31578>.
- 964 [121] Yuen M-F, Heo J, Jang JW, Yoon J-H, Kweon YO, Park S-J, et al. Hepatitis B virus (HBV)  
965 surface antigen (HBsAg) inhibition with isis 505358 in chronic hepatitis B (CHB)  
966 patients on stable nucleos (t)ide analogue (NA) regimen and in NA-naive CHB  
967 patients: phase 2a, randomized, double-blind, placebo-controlled study. *Journal of*  
968 *Hepatology* 2020;73:S49–50. [https://doi.org/10.1016/S0168-8278\(20\)30646-2](https://doi.org/10.1016/S0168-8278(20)30646-2).
- 969 [122] Yuen M-F, Locarnini S, Given B, Schluep T, Hamilton J, Biermer M, et al. First clinical  
970 experience with RNA interference-based triple combination therapy in chronic  
971 hepatitis B: JNJ-3989, JNJ-6379 and a Nucleos (t) ide analogue. *Hepatology*  
972 2019;70:1489A.
- 973 [123] Wedemeyer H, Schöneweis K, Bogomolov PO, Chulanov V, Stepanova T, Viacheslav M,  
974 et al. 48 weeks of high dose (10 mg) bulevirtide as monotherapy or with  
975 peginterferon alfa-2a in patients with chronic HBV/HDV co-infection. *Journal of*  
976 *Hepatology* 2020;73:S52–3. [https://doi.org/10.1016/S0168-8278\(20\)30651-6](https://doi.org/10.1016/S0168-8278(20)30651-6).
- 977 [124] Bazinet M, Pântea V, Placinta G, Moscalu I, Ceboatarescu V, Cojuhari L, et al. Safety and  
978 Efficacy of 48 Weeks REP 2139 or REP 2165, Tenofovir Disoproxil, and Pegylated

979 Interferon Alfa-2a in Patients With Chronic HBV Infection Naïve to Nucleos(t)ide  
980 Therapy. *Gastroenterology* 2020;158:2180–94.  
981 <https://doi.org/10.1053/j.gastro.2020.02.058>.

[125] Gane E, Dunbar PR, Brooks A, Zhao Y, Tan S, Lau A, et al. Efficacy and safety of 24  
982 weeks treatment with oral TLR8 agonist, selgantolimod, in virally-suppressed adult  
983 patients with chronic hepatitis B: a phase 2 study. *Journal of Hepatology* 2020;73:S52.  
984 [https://doi.org/10.1016/S0168-8278\(20\)30650-4](https://doi.org/10.1016/S0168-8278(20)30650-4).

[126] Michler T, Kosinska AD, Festag J, Bunse T, Su J, Ringelhan M, et al. Knockdown of Virus  
985 Antigen Expression Increases Therapeutic Vaccine Efficacy in High-Titer Hepatitis B  
986 Virus Carrier Mice. *Gastroenterology* 2020;158:1762-1775.e9.  
987 <https://doi.org/10.1053/j.gastro.2020.01.032>.

[127] Gane E, Verdon DJ, Brooks AE, Gaggar A, Nguyen AH, Subramanian GM, et al. Anti-PD-  
988 1 blockade with nivolumab with and without therapeutic vaccination for virally  
989 suppressed chronic hepatitis B: A pilot study. *Journal of Hepatology* 2019;71:900–7.  
990 <https://doi.org/10.1016/j.jhep.2019.06.028>.

[128] Maini MK, Pallett LJ. Defective T-cell immunity in hepatitis B virus infection: why  
991 therapeutic vaccination needs a helping hand. *The Lancet Gastroenterology &*  
992 *Hepatology* 2018;3:192–202. [https://doi.org/10.1016/S2468-1253\(18\)30007-4](https://doi.org/10.1016/S2468-1253(18)30007-4).

[129] Dandri M. Epigenetic modulation in chronic hepatitis B virus infection. *Semin*  
993 *Immunopathol* 2020;42:173–85. <https://doi.org/10.1007/s00281-020-00780-6>.

[130] Revall PA, Chisari FV, Block JM, Dandri M, Gehring AJ, Guo H, et al. A global scientific  
994 strategy to cure hepatitis B. *The Lancet Gastroenterology & Hepatology* 2019;4:545–  
995 58. [https://doi.org/10.1016/S2468-1253\(19\)30119-0](https://doi.org/10.1016/S2468-1253(19)30119-0).

1000  
1001  
1002  
1003  
1004  
1005  
1006  
1007  
1008  
1009

1010 **FIGURES LEGENDS**

1011

1012 **Figure 1. cccDNA biogenesis** requires removal of the viral polymerase (POL) generating  
1013 protein-free rcDNA (PF-rcDNA, aka DP-rcDNA). The DNA flap and RNA primer should be  
1014 eliminated and the single stranded DNA gap in the plus strand repaired to obtain the  
1015 cccDNA molecule. A non-exhaustive list of the enzymes from the host-repair system  
1016 potentially involved in cccDNA biogenesis are indicated in each step.

1017

1018 **Figure 2. cccDNA kinetics and stability.** A dynamic model, summarized in the left panel  
1019 assumes constant cccDNA degradation and formation. Both Intracellular recycling of rcDNA  
1020 to the nuclei of infected cells to form novel cccDNA and *de novo* infection could contribute  
1021 to the maintenance of the cccDNA pool while the mechanisms ruling its degradation are not  
1022 currently known. On the right panel a static model of cccDNA maintenance is schemed in  
1023 which nuclear cccDNA is stable. Thus, rcDNA recycling and *de novo* infections are not  
1024 essential to maintain the cccDNA pool. In both models, cell division can lead to cccDNA  
1025 dilution thus reducing its levels in the infected liver.

1026

1027 **Figure 3. Novel cccDNA targeting approaches and their expected mechanism of action.**

1028 A- Irreversible cccDNA silencing or degradation would lead to a sustained reduction of  
1029 pgRNA and viral antigen production.

1030 B- Permanent cccDNA alterations inducing lethal mutations leading to suppression of viral  
1031 antigen production. In this scenario cccDNA levels will not be directly affected, unless  
1032 high liver inflammation leads to increased cell division “diluting” cccDNA.

1033 C- Reversible cccDNA silencing leading to low viral antigen production intervals that could  
1034 allow T-cell restauration. Once the HBV specific CTL are reestablished it is conceivable  
1035 that they could control the infection, although it is possible that a CTL boost may be  
1036 necessary.

1037

1038

1039

1040

1041

1042

1043

1044

1045

1046

1047

1048

1049

1050

1051

1052

1053

1054

1055

1056



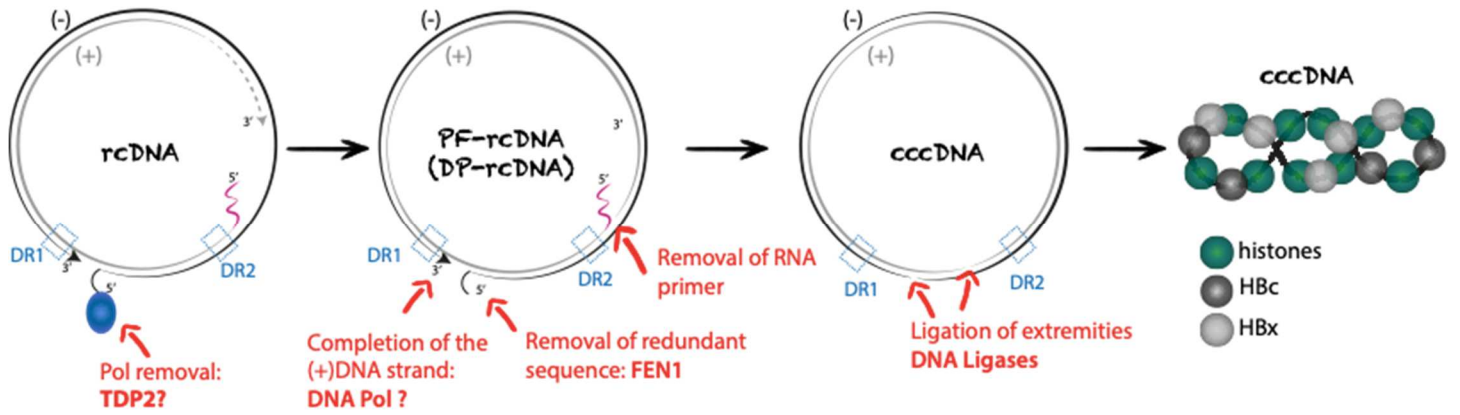
1057

1058 **FIGURES**

1059

1060 **Figure 1**

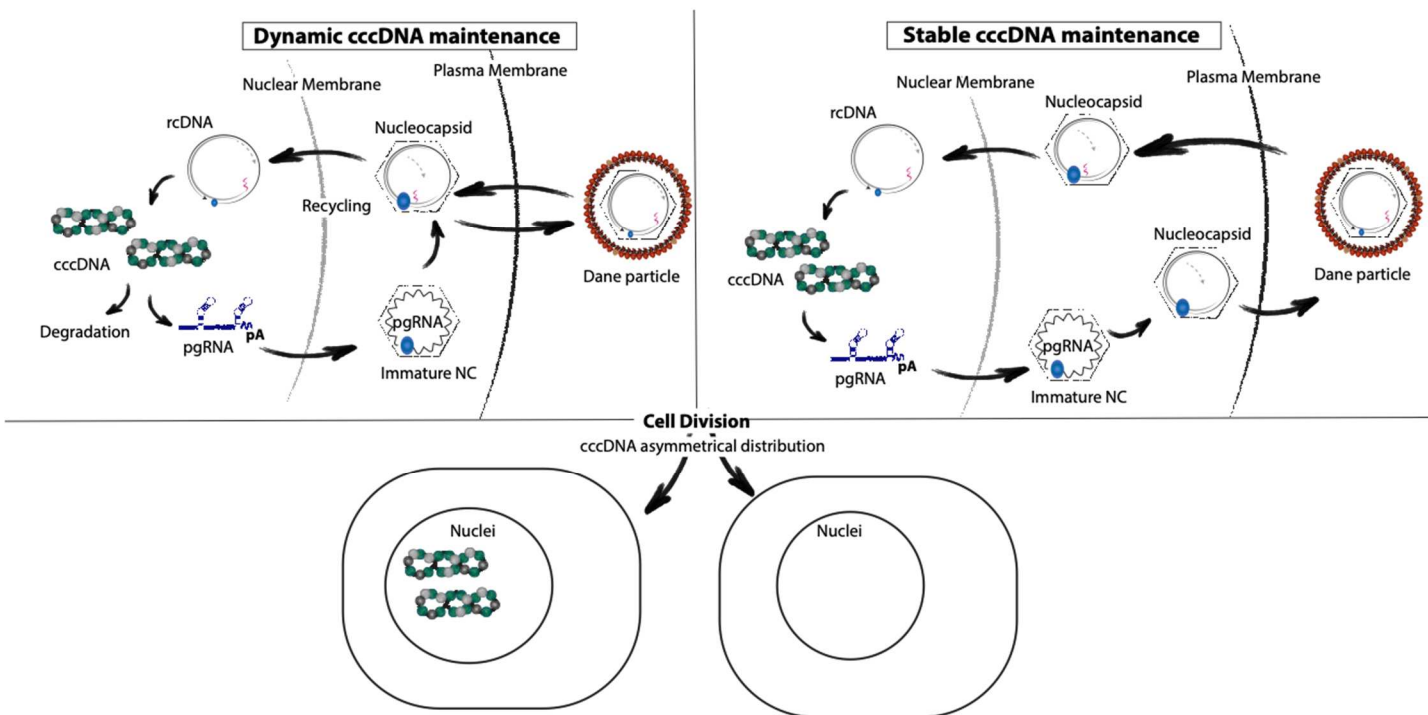
1061



1062

1063

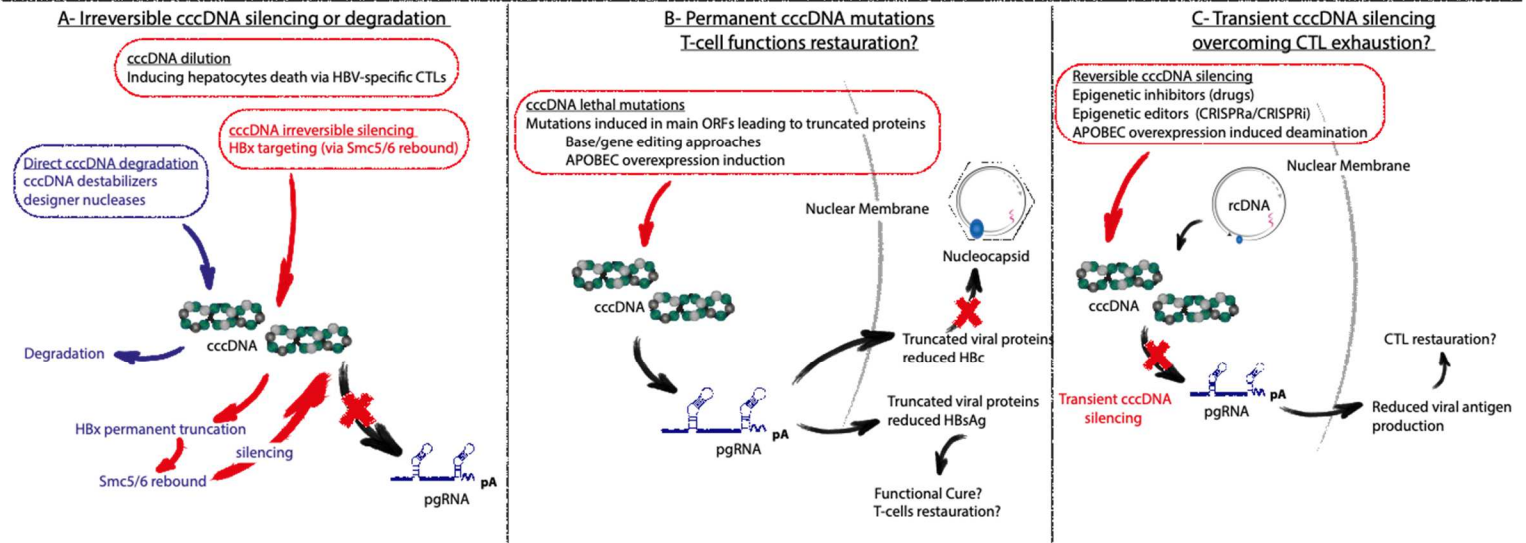
1064 **Figure 2**



1065

1066

**Direct cccDNA targeting strategies**



1067 **Figure 3**